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(54) **Ligand gold bonding**

Bindung von Liganden an Gold

Ligand reliant des particules d'or

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Description**BACKGROUND:**

5 This invention relates to gold sol coated with alkanethiols and alkanethiol derivatives to provide groups on the sol available for the binding or linking of binding moieties such as antibodies, antigens, or ligands to the gold sol. In addition, the use of di- and tri-thiol compounds bound to gold sol facilitate the passive adsorption of these binding moieties. This invention also relates to gold sol coated with thiolated binding moieties, including antigens, antibodies, or carrier molecules, which can be attached to relevant ligands. Also included is the process for coating the gold sols with such thiol compounds, the use of coated sols in immunological and immunocytological diagnostic tests and test kits incorporating such coated gold sols.

Test methods for the diagnosis of various diseases are constantly being improved. Currently, immunological methods are among the most sensitive methods used to detect the presence of antigens or antibodies in samples. These assays are well known to those skilled in the art of immunodiagnosics. Generally, an immunological assay consists of an assay wherein a monoclonal or polyclonal antibody is used to capture an antigen in the sample and a second antibody containing a label, such as a fluorescent compound or an enzyme, immunochemically reacts with the antigen-antibody complex. The resulting labelled antibody-antigen-antibody complex is detected. Variations on this basic assay are common, such as the use of only one reactive antibody in the test, the competitive inhibition method, or the use of particles as labels that allow an agglutination reaction to be read. Another variant is the use of microparticles coated with either an antigen or an antibody, which after formation of a complex with the analyte and a second appropriately labeled binding partner, gives a positive reaction.

Gold sol microparticles are used in an assay method known as a sol particle immunoassay (SPIA). In this assay, a solution containing the gold sol coated with an appropriate binding partner, either an antibody or an antigen, is reacted with a sample to bind to its binding partner. In this process, complexes are formed that can be detected, usually due to their change in color.

Uncoated gold sol particles, and other colloids, will undergo agglomeration when exposed to low concentrations of salt, and quickly precipitate out of solution. Therefore, the coating of gold sol with an appropriate binding partner serves two functions. The first is to provide appropriate immunological binding activity and the second is to protect against agglomeration, which would of necessity occur in buffers designed to optimize for immunological reactions. Since not all proteins or polymers will protect the gold sol completely from salt-induced agglomeration, an overcoating step is usually performed with a protein or polymer that is known to be capable of completely protecting the gold sol from salt-induced agglomeration. Such an overcoating step is a well known practice in adsorbing antibodies and antigens to plastic substrates and has been shown to increase the stability of the coated material.

The overcoating also serves to reduce nonspecific interaction of the gold sol with sample components. This nonspecific interaction is a significant problem when using antibody coated latex particles in diagnostic assays, and is probably a manifestation of the nonspecific serum interference observed in many, if not all, immunoassays, regardless of format. In some cases it is permissible to change the overcoating protein or polymer to minimize interference in specific systems. Additives to the sol medium such as guanidine hydrochloride or urea are useful. Occasionally, "non-specific" interference can be pinpointed to serum heterophile activity and is eliminated by the addition of whole animal serum.

A serious drawback in the passive adsorption of the desired binding partner to gold colloid has been that such direct coating is often unsuccessful. The physico-chemical mechanisms of passive polymer adsorption to colloids in general is a poorly understood process. Passive adsorption of antibodies to gold sol may result in a coated sol which is poorly protected from salt-induced agglomeration and which cannot be further protected by overcoating. It may also result in a coated sol with poor immunological activity, presumably due to the incorrect orientation of adsorbed antibody, or it may result in the antibody-induced agglomeration of the sol itself. Finally, the binding partner of choice may simply not bind to the gold sol. The net result of these problems is that few biological reagents useful in other diagnostic formats can be used in the production of gold colloid reagents of diagnostic quality.

What is needed in the art is a method for covalently attaching binding partners, proteins, carbohydrates or ligands, to the gold sol so that the uncertainties of the passive adsorption characteristics or the necessity of making binding partner and "good coating" carrier conjugates for passive adsorption may be eliminated. In particular, such a sol would be significantly more useful if it were refractive to salt-induced agglomeration even in the absence of a coated binding partner. The ability to change the physico-chemical surface properties of the sol would, at the same time, make it possible to minimize the sometimes undefined sample-sol interactions responsible for non-specific interference in immunochemical diagnostic assays and background problems in immunocytochemical assays.

What is also needed is the ability to facilitate the passive adsorption of biological polymers to gold sols. Thiolation of antibodies and polymers can increase their ability to bind to gold solo as evidenced by increased resistance to salt-induced agglomeration. Such a capability may prove useful for those antibodies which bind well to gold sols, but lose

significant amounts of their activity in doing so as well as for antibodies which simply do not bind the gold sol in an underivatized state. An alternative method of changing the physico-chemical characteristics of the sol surface in order to facilitate antibody binding is the coating of the sol with di-thiol or tri-thiol compounds. Such an intermediate coating can significantly change the passive adsorption properties of antibodies to the coated sol.

SUMMARY OF THE INVENTION

This invention provides a process for coating microparticles of gold sol with alkanethiols, alkanethiol derivatives, and di- and tri-thiol compounds. Gold sols coated with alkanethiols or their derivatives are resistant to salt-induced agglomeration and contain chemical moieties for covalent polymer or ligand attachment. A particular hydrophobic-hydrophilic balance of the sol surface is obtained and nonspecific interactions of the sol with proteins generally are minimized. In particular, chemical groups distal to the n-alkane thiol moiety, such as methyl, hydroxyl, carboxyl, amino, sulfhydryl or carbonyl, are solvent exposed and serve as covalent attachment sites and may be hydrophobic-hydrophilic balance sites.

Coating gold sol with small molecular weight di- and tri-thiol compounds does not protect the sol from salt-induced agglomeration, but changes the physico-chemical nature of the coated sol surface and facilitates the passive adsorption of antibody molecules.

Also included in the present invention is the coated gold sol. The invention also includes coated gold sol particles additionally bound to binding moieties such as antibodies or antigens and diagnostic kits containing said gold sol particles.

Lastly, the invention includes coated gold sols to which binding moieties are attached by adsorption, and uncoated gold sol where adsorption is facilitated through the thiolation of the binding moiety.

DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the relative resistance to salt-induced gold sol agglomeration conferred by coating the gold sol with anti-gp160 antibody (GC-143) at various concentrations and pH values.

Figure 2 demonstrates the relative resistance to salt-induced gold sol agglomeration conferred by coating a TTC coated gold sol with anti-gp160 antibody (GC-143) at various concentrations and pH values.

Figure 3 demonstrates the ability of GC-143 to cause spontaneous agglomeration of a gold sol in the absence of any added salt at various concentrations and pH levels.

Figure 4 demonstrates the ability of GC-143 to cause spontaneous agglomeration of a TTC coated gold sol in the absence of any added salt at various concentrations and pH levels.

Figure 5 shows the immunological activity and specificity of GC-143 adsorbed on a gold sol in a SPIA assay.

Figure 6 shows the immunological activity and specificity of GC-143 adsorbed on a TTC coated gold sol in a SPIA assay.

Figure 7 demonstrates the ability of an underivatized monoclonal anti-HIV p24 antibody adsorbed to gold sol to protect the sol from salt-induced agglomeration.

Figure 8 demonstrates the ability of thiolated monoclonal anti-HIV p24 antibody adsorbed to gold sol to protect the sol from salt-induced agglomeration.

DETAILED DESCRIPTION OF THE INVENTION

The process of coating microparticles consisting of gold sol to provide chemical linking groups to facilitate the further attachment of other groups is the basis of the present invention. Microparticles in the invention are generally defined as colloidal gold sol that range in size from about 20nm to about 200nm or more. The preferred range of microparticle is from about 60 to about 80nm. The most preferred size is about 65 to 75nm. Some of the coating chemicals used, such as alkanethiols and their derivatives and mixtures thereof also protect the gold sols from salt-induced agglomeration and may produce a particular hydrophobic-hydrophilic balance of the sol surface so that non-specific interactions of the surface with extraneous proteins are minimized.

Gold sol was produced by the hydroxylamine-mediated reduction of chloroauric acid in water onto seed gold particles. This procedure is described in the literature. (Turkevich, J. et al., Discussions of the Faraday Society, No. 11, p. 55-74 (1951)). Generally, chloroauric acid trihydrate dissolved in water is added to deionized water, to which is then added hydroxylamine hydrochloride. Seed gold particles are added and stirred. A small amount of acetone is added, and the mixture is stirred. K_2CO_3 is added until the pH reaches 7.0. The gold sol ranges in size from about 10nm to about 150nm in diameter depending on the amounts of gold particle added and the amount of chloroauric acid used, and is now ready to be coated with alkanethiols or thiol derivatives.

Alkanethiols, their derivatives, and di- and tri-thiol compounds are the preferred compounds used to coat the gold

sol particle and to provide chemical moieties or linking groups for covalent polymer or ligand attachment to the gold sol or for the modification of the hydrophilic/hydrophobic balance. The more preferred are the n-alkanethiols, while the most preferred compounds are alkanethiols of the formula $\text{CH}_3(\text{CH}_2)_n\text{SH}$, where n equals 9 to 23. Derivatives of the alkanethiols are generally of the formula $\text{RCH}_2(\text{CH}_2)_n\text{SH}$ wherein R is OH, COOH, CHO, SH and NH_2 , and n is 9 to 23. The di- and tri-thiol compounds are generally compounds of low molecular weight. Some of them have been recognized as heavy metal chelators. Two examples are 2,3-dimercaptosuccinic acid and trithiocyanuric acid.

To coat a sol with an n-alkanethiol, 0.10M of the alkanethiol and 1% Tween-20^R (ICI Americas registered trademark, polyoxyethylenesorbitan monolaurate) in alcohol, preferably methanol, is freshly prepared. Approximately 1ml of this mixture is added to approximately 100ml of the prepared gold sol, and is stirred. The resulting mixture is allowed to set at room temperature for approximately 2 hours. Lower concentrations of the coating compound will effectively coat gold sol but the optimal coating times must be found empirically for each case.

The sol mixture is washed into an approximately 1mM 3-[N-Morpholino]-2-hydroxypropane sulfonic acid (MOPSO), pH 7 buffer by centrifugation at 2000 x g for approximately 15 minutes at room temperature, and can be stored at 4°C until use.

The above is an example of coating gold sol with an n-alkanethiol compound. This coating procedure is modified for the attachment of mixtures of n-alkanethiols and n-alkanethiol derivatives containing linking groups for the chemical conjugation of ligands, or polymeric compounds. For example, the inclusion of an hydroxy-n-alkanethiol to the coating mixture increases the hydrophilicity of the sol surface and decreases the non-specific interaction of the sol with serum proteins. The inclusion of a carboxyl-n-alkanethiol at a level of 10% of total thiol compounds present is sufficient to produce a sol with the desired density of conjugation moieties. The carbon chain length of the n-alkanethiol in these mixtures can be 2-4 carbons less than the carbon chain length of the n-alkanethiol derivatives in order to better expose the conjugation moieties to reduce steric hindrance associated with reaction of macromolecules with the sol surface.

The coating solution of n-alkanethiol, n-alkanethiol derivative or mixtures of the two groups is generally made in alcohol, preferably methanol, with 1% Tween-20^R. The total concentration of all the thiol compounds is generally no greater than 0.10M. In some cases, the optimal pH for coating thiol compounds is not 7. For example, in the case of trithiocyanuric acid coated sols, the sol is adjusted to pH 6 before the addition of the thiol compound to optimize the degree of resistance to salt-induced agglomeration conferred by a particular thiol compound mixture.

The sols are generally allowed to coat in the presence of the thiol mixture for 2 hours, but optimal incubation times are determined empirically for each case. This is accomplished by testing the sols for their resistance to salt-induced agglomeration after incubation periods of varying length using a spectrophotometric assay. Gold sols have a characteristic violet color, which is exceedingly sensitive to the diameter of the sol particles. If sol particle agglomeration takes place, the effective "size" of the sol is greatly increased and the characteristic absorption maximum shifts from 540nm, which is violet, to higher wavelengths, with a blue to colorless sol color.

In practice, 1ml of the coated sol aliquot is dispensed into a glass tube. One hundred microliters of 10% NaCl are added to the sol aliquot with mixing. The mixture is allowed to set at ambient temperature for 10 minutes and its absorbance at 540nm is compared to an identical sol aliquot to which 100 microliters of water have been added. A test aliquot that retains 90% or greater of the absorbance of the control aliquot is considered to be resistant to salt-induced agglomeration.

Compounds tested are shown in Table 1 below.

TABLE 1

Compound(s)	Relative Degree of Protection From Salt-Induced Agglomeration*
n-hexadecanethiol	97%
n-dodecanethiol	93%
n-hexadecane	25%
1-hexadecanoic acid	26%
12-mercapto-1-dodecanoic acid	46%
11-mercapto-1-undecanol	1
2,3-dimercapto succinic acid	41%
2,5-dimercapto-1,3,4-thiadiazole	94% ²
3-amino-5-mercapto-1,2,4-triazole	<30%
trithiocyanuric acid	<30%
n-dodecanethiol:	
12 mercaptododecanoic acid	
(1:1) ³	74%
(4:1)	76%
(9:1)	90%
n-decanethiol:11-mercapto-1-undecanol:	
12-mercapto dodecanoic acid	
(8:1:1)	100%
(8:3:1)	83%

*expressed as % optical density at 540nm of control sol aliquot with no added salt.

¹ the sol was spontaneously agglomerated in the presence of the coating compound

² even though the sol was protected from salt-induced agglomeration, after harvesting by centrifugation the sol was no longer salt protected.

³ mole ratios of compounds used in coating mixtures

The compounds found to be most successful in coating the gold sol and protecting it from salt-induced agglomeration were n-alkanethiols. N-alkanethiol derivatives such as 12-mercapto-1-dodecanoic acid were ineffective by themselves in conferring resistance to salt-induced agglomeration, but sols coated with mixtures of n-alkanethiols and their derivatives did confer complete protection from salt-induced agglomeration and contributed the other properties discussed above.

The coated gold sol containing chemically conjugatable groups is then bound to binding moieties such as proteins, carbohydrates, antibodies, antigens, polymers, monomers or ligands using carbodiimide chemistry by well known methods. Binding moieties include any groups that are capable of being adsorbed or covalently attached to the coated gold sol. In some cases, carbodiimide chemistry is also used to conjugate a linker molecule such as 6-amino caproic acid to the sol prior to the indirect conjugation of the binding partner in order to relieve steric constraints on the binding partner and aid in its recognition by and of its complementary binding partner. In order to conjugate the binding partner or the linker molecule to the coated sol, the sol is incubated at ambient temperature in the presence of the substance to be coupled, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride(EDC), and N-hydroxysulfosuccinimide (SNHS), in a 10-25mM buffer at pH 7.0 for 2 hours. In some cases, sequential aliquots of EDC are added during the conjugation reaction in order to compensate for the hydrolysis of this compound in aqueous media.

The choice of potentially useful conjugation chemistries is determined by the chemical moiety on the coated sol as well as by the type of moiety introduced on any given linker molecule. For example, amino groups on the sol-bound alkanethiol derivatives or attached linker molecules could be conjugated to polymers using homo- or hetero-bifunctional N-hydroxysuccinimide ester crosslinkers. Likewise, sulfhydryl groups on sol-bound alkanethiol derivatives or linker molecules could be conjugated to polymers using homo- or hetero-bifunctional maleimide crosslinkers. The range of possible conjugation schemes is very similar, if not identical to, the range of chemical schemes currently used in the preparation of diagnostic immunoconjugates. Classical crosslinking reagents compatible with the moieties on the sol, the alkanethiol derivatives or the chemical crosslinkers are needed to attach the appropriate binding partner to the sol.

Immunoassays using the sol particle immunoassay (SPIA) method are performed as described in US Patent 4,313,734 to J. Leuvering. Briefly, gold sol coated with a binding moiety is incubated in the presence of a test sample containing the opposite binding partner. If the opposite binding partner is present in the sample, cross linking of the

coated sol will result and the absorbance at the absorption maximum of single gold sol particles will decrease dramatically. The adsorption properties of polymeric compounds to gold sols may be facilitated by the thiolation of the binding partner.

Both polyclonal and monoclonal antibodies were thiolated by reaction with N-succinimidyl S-acetylthioacetate (SATA). The resulting protected sulfhydryl groups were exposed by reaction of the proteins with hydroxylamine hydrochloride. This is a well known chemical procedure for introducing sulfhydryl groups into protein. The thiolated proteins were then adsorbed to gold sols at pH's 6, 7, 8 and 9 at protein concentrations ranging from 5µg/ml to 100µg/ml. The degree of resistance to salt-induced agglomeration was determined as described above. Thiolation of antibodies by this method significantly improved their ability to protect gold sol from salt-induced agglomeration over non-thiolated antibodies, as shown in Figures 7 and 8, and described in Example 12.

An alternative method of facilitating the adsorption of antibodies to gold sol is the inclusion of an intermediate coating between the gold sol and the binding partner in order to change the physico-chemical properties of the sol surface such that adsorption of the antibodies is increased. Di- and tri-thiols are used for such a purpose. Trithiocyanuric acid (TTC) is an example of such a compound. Gold sol was coated with TTC in a procedure similar to that described above for the n-alkanethiol compounds but with different concentrations of TTC and Tween-20^R. These concentrations were determined empirically. The ability of antibody to confer resistance to salt-induced agglomeration of the washed sol was determined after incubation of the antibody and the coated sol at various pH's and protein concentrations in the previous paragraph.

The coated microparticles of this invention may be covalently bound to antigen or antibodies and sold in a test kit for use in immunodiagnostics. The antigen or antibody will be specific for the opposite binding partner sought to be detected, and the other components of the kits may include diluents, buffers, labeling reagents and laboratory equipment that will also be specific for the particular test to be performed.

EXAMPLES

Example 1. Preparation of Gold Sol.

For preparation of the seed sol used in the final gold sol preparation, 10ml of 1% HAuCl₄ trihydrate was added to 900 ml of water and stirred well. 10ml of 1% sodium citrate was added to the mixture and stirred well. With vigorous stirring, 10ml of 0.075% sodium borohydride was added (made by dissolving sodium borohydride in the sodium citrate solution) and stirred well for 5 minutes. The mixture was filtered through a 0.22 micron filter and stored at 4°C. The sol should age at least 24 hours before use.

For preparation of the final gold sol, 8ml of 2% HAuCl₄ trihydrate (in water) was added to 900ml of water. With continuous stirring, 4ml of freshly prepared 5% hydroxylamine hydrochloride was added (in water). Immediately thereafter, 22.33 microliters of seed sol was added and stirred vigorously for 30 minutes. 1 microliter of acetone was added for each milliliter of sol produced and stirring continued for 10 minutes. 0.2M K₂CO₃ was added until the desired pH was obtained.

The appropriate volume of seed sol added was determined by the following relationship: Volume of seed required for desired sol size = Volume of seed required for sol of given size times the cube root of (Diameter of sol obtained from a given amount of seed sol divided by diameter of the desired particle).

Example 2. Coating of Gold Sol with n-Alkanethiol.

Gold sol was prepared as described in Example 1. A solution of 0.1M 1-dodecanethiol in methanol and 1% Tween-20^R was freshly prepared. For every 100ml of gold sol to be coated, 1ml of the thiol-methanol solution was added dropwise with stirring to the gold sol. The mixture was allowed to set at ambient temperature with occasional swirling for 2 hours. The coated gold sol was recovered by centrifugation at 2000 x g for 15 minutes at ambient temperature with subsequent resuspension in 1mM MOPSO, pH 7.0. This centrifugation and resuspension was repeated 3 times. The resultant sol was resuspended in the same buffer and stored at 4°C prior to use.

Example 3. A n-Alkanethiol and n-Alkanethiol Derivative Mixture Coated Gold Sol.

Gold sol was prepared as described in Example 1. A solution of 0.09M 1-dodecanethiol, 0.01M 12-mercapto-1-dodecanoic acid in methanol and 1% Tween-20^R was freshly prepared. For every 100ml of gold sol to be coated, one ml of the thiol mixture-methanol solution was added dropwise with stirring to the gold sol. The mixture was allowed to set at ambient temperature with occasional swirling for 2 hours. The coated gold sol was recovered in a manner identical to the previous example.

Example 4. n-Alkanethiol Derivative Gold Sol Coating and Subsequent Lack of Protection from Salt-Induced Agglomeration.

Gold sol was prepared as described in Example 1 and adjusted to pH 7 with K_2CO_3 . A solution of 0.10M 12-mercapto-1-dodecanoic acid and 1% Tween-20^R in methanol was freshly prepared. For every 100ml of gold sol to be coated, 1ml of the thiol-methanol solution was added dropwise with stirring to the gold Sol. The mixture was allowed to set at ambient temperature with occasional swirling for 1 hour. To duplicate 1ml aliquots of the coated sol were added either 100 μ l of water or 10% NaCl. The aliquot to which water was added was considered the control. The aliquot to which 104 NaCl was added had lost 54% of its optical density after 10 minutes. This is interpreted as evidence that the gold sol was not protected from salt-induced agglomeration by being coated with the 12-mercapto-1-dodecanoic acid.

Example 5. Preparation of a Gold Sol Coated with a Mixture of n-Alkanethiol and n-Alkanethiol Derivatives.

Gold sol was prepared as described in Example 1 and adjusted to pH 7 with K_2CO_3 . A solution of 0.004M N-decanethiol, 0.0005M 11-mercapto-1-undecanol, 0.0005M 12-mercapto-1-dodecanoic acid, 0.08M NaCl and 1% Tween-20^R in methanol was freshly prepared. For every 100ml of sol to be coated, 1ml of the thiol-thiol derivative solution was added dropwise with stirring and the mixture was allowed to set overnight at ambient temperature. The coated sol was assayed for protection from salt-induced agglomeration as in Example 4.

The coated sol retained 100% of its optical density at 540nm after the addition of 10% NaCl. This is believed to show that the coated sol is fully protected from salt-induced agglomeration.

Example 6. Bovine Serum Albumin (BSA) Conjugation to Coated Gold Sol.

Four ml of the coated gold sol described in Example 3 with an optical density at 540nm of 25.0 was mixed with 4.3ml of 10mM MOPSO, pH 7.0. To this mixture was added sequentially with stirring, 0.2ml of 25mg/ml BSA, 1.0ml of 30mM SNHS, and 0.5ml of 0.1M EDC. The BSA, SNHS and EDC were prepared in 10mM MOPSO, pH 7.0. The reaction was rocked for 2 hours at ambient temperature. The reaction was quenched by an additional hour of rocking after the addition of 1ml of 1.0M ethanolamine (adjusted to pH 7.0 with HCl), pH 7.0 in 10mM MOPSO. An equal volume of 10mM MOPSO, 0.15M NaCl, 0.5% Tween-20^R, 1.0mg/ml casein, at pH 7.0 was added to the reaction mixture and the mixture was allowed to set for 1 hour. This blocking or overcoating step protects the unreacted bare areas of the gold sol surface from nonspecific interaction with other biopolymers. The BSA conjugated coated sol was recovered by centrifugation at 2000 x g at ambient temperature for 15 minutes and subsequent resuspension in the same buffer. This centrifugation and wash was repeated 3 times. The sol was centrifuged as above and resuspended in 10mM MOPSO, pH 7.0. This step was repeated twice. The BSA conjugated coated sol was stored at 4°C prior to use.

Example 7. SPIA of BSA Conjugated Gold Sol.

The BSA conjugated gold sol from Example 5 was diluted in 0.1M MOPSO, 0.15M NaCl, 1.0% polyethylene glycol 8000, pH 7.0 to obtain an optical density at 540nm of 1.0. Polyclonal anti-BSA antibody and normal rabbit immunoglobulin(IgG) were diluted in 10mM MOPSO, pH 7.0 to 12 microliters/ml. To separate 1ml aliquots of BSA conjugated sol were added 100 microliters of the diluted anti-BSA or normal rabbit IgG, or 10mM MOPSO buffer with mixing. The mixtures were transferred to cuvettes and the optical densities at 540nm were monitored over time and shown in Table 2.

The results in Table 2 demonstrate that there is immunologically recognizable BSA on the surface of the sol and that the decrease in optical density is not due to non-specific interaction of rabbit serum with the sol.

Example 8. SPIA of BSA Negative Control Conjugated Gold Sol.

A negative control BSA conjugated coated sol was prepared exactly as in Example 5 above except that no EDC was added to the conjugation reaction. An identical volume of 10mM MOPSO, pH 7.0 buffer was included instead. The purpose of this sol was to demonstrate in SPIA assays that the BSA activity observed was covalent in nature. Comparison of results in Table 2 and 3 demonstrate that although a significant portion of the BSA activity on these sols may be passively adsorbed, an equally significant portion is covalently bound.

TABLE 2

SAMPLE	BSA CONJUGATED COATED SOL DECREASE IN O.D. 540nm AT 60'
Anti-BSA	0.401

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TABLE 2 (continued)

SAMPLE	BSA CONJUGATED COATED SOL DECREASE IN O.D. 540nm AT 60'
Rabbit IgG	0.029
Buffer	0.031

TABLE 3

SAMPLE	BSA NEGATIVE CONTROL CONJUGATED GOLD SOL DECREASE IN O.D. 540nm AT 60'
Anti-BSA	0.238
Rabbit IgG	0.063
Buffer	0.050

Example 9. Improvement in Coating Properties of Antibody on TTC Coated Gold Sol.

Gold sol was prepared as described in Example 1, except that its pH was adjusted to 6.0 with K_2CO_3 . A saturated solution of TTC in methanol was freshly prepared. A solution of 10% Tween-20^R in methanol was added until the final Tween-20^R concentration was 2.5%. Four ml of this solution was added to 200ml of the above sol with stirring and the mixture was allowed to set at ambient temperature for 2 hours with occasional swirling. The coated sol was harvested as in Example 2. Separate aliquots of the coated sol were diluted to an optical density of 2.0 and simultaneously adjusted to pH 6, 7, 8 and 9 with 10mM (2-[N-morpholino]ethanesulfonic acid) (MES), 10mM MOPSO, 10mM (N-[2-hydroxyethyl]-piperazine-N-[3-propanesulfonic acid]) (EPPS), and 10mM (2-[N-cyclohexylamino]-ethanesulfonic acid) (CHES), respectively. In parallel, a gold sol was prepared as in Example 1 and separate aliquots were adjusted to pH 6, 7, 8 and 9 with K_2CO_3 . A purified human anti-HIV polyclonal antibody was diluted to 1.0, 0.5, 0.25, 0.10 and 0.05mg/ml in each of the above buffers. To parallel 1.0ml aliquots of each of the above sols and each pH was added 100 μ l of antibody solution at the corresponding pH with mixing. The mixtures were allowed to set at ambient temperature for 30 minutes. One hundred μ l of 10% NaCl was added to each sol mixture with agitation. After an additional 10 minutes, the optical density at 540nm of each mixture was measured, as shown in Figures 1 - 4.

The adsorption of the antibody on the TTC coated sol resulted in a broader pH range at which protection from salt-induced agglomeration occurred. Also, protection from salt-induced agglomeration took place at lower antibody concentrations on the TTC coated sol. In the absence of NaCl addition, the antibody caused spontaneous agglomeration of the uncoated sol. The antibody-induced spontaneous agglomeration was much less apparent on the TTC coated sol.

Collectively, these observations show that the adsorption of this antibody on TTC coated gold sol is greater than that seen on uncoated sol.

Example 10. Improvement in SPIA Activity of Antibody Adsorbed on TTC Coated Gold Sol.

One hundred ml of gold sol was prepared as in Example 1 and the pH was adjusted to 9.0. Two and one-half milliliters of 2mg/ml of the human anti-HIV (GC-143) in 10mM CHES, pH 9.0 was added with stirring and the mixture was allowed to set at ambient temperature for approximately 2 hours with occasional swirling. Five ml of 1mg/ml nonfat dry milk was added to the mixture with stirring and the mixture was allowed to set at ambient temperature for an additional hour. The coated sol was centrifuged at 2000 x g for 15 minutes at ambient temperature and resuspended in 10mM CHES, 0.5% BSA, 300mM mannitol, 0.01% sodium aside, at pH 9.0. The centrifugation and resuspension step was repeated 3 times. The sol was finally resuspended in the same buffer and stored at 4°C prior to use. A TTC coated sol prepared as in Example 9 was diluted with 10mM CHES at pH 9.0 to obtain 60ml of sol with an optical density of 2.0, the same approximate optical density of the gold sol prepared in Example 1. Six ml of 0.5mg/ml human anti-HIV dissolved in 10mM CHES, pH 9.0 was added with stirring and the mixture was allowed to set at ambient temperature for 1 hour, then overnight at 4°C. Six ml of 1mg/ml nonfat dry milk was added with stirring and the mixture was allowed to set at ambient temperature for 1 hour. The coated sol was harvested as above and stored identically.

Aliquots of each sol were diluted to an optical density at 540nm of 2.0 with 0.1M MOPSO, 0.15M NaCl, 1% PEG 8000, 0.25% BSA, at pH 7.0. To replicate aliquots of each diluted sol were added a) one hundred μ l buffer, b) one hundred μ l of 10 μ g/ml HIV gp160, or c) one hundred μ l of a mixture of 5 μ g/ml HIV gp160 and 5 μ g/ml human anti-HIV which had been pre-incubated for 90 minutes at ambient temperature, as shown in Figures 5 and 6.

It is apparent that the GC-143 TTC coated sol is more immunologically active than its uncoated sol counterpart and that its use in the SPIA assay results in a more sensitive assay. Therefore, by functional criteria as well, the GC-143 TTC coated sol is also better than the uncoated sol counterpart.

Example 11. Thiolation of Monoclonal Anti-HIV p24 using N-succinimidyl-S-acetylthioacetate (SATA).

One ml of monoclonal anti-HIV p24 at 1.46mg/ml in 50mM NaPO₄, 1mM ethylenediamine tetraacetic acid (EDTA), pH 7.5 was mixed with 0.02ml of 5mg/ml SATA in dimethylsulfoxide (DMSO) and allowed to react at ambient temperature for 2 hours. The reacted antibody was separated from the reaction components by gel filtration through a desalting column in the same buffer. One quarter mg of the reacted gel filtered antibody was diluted to 1 ml with the same buffer and 100µl of 0.05M NaPO₄, 25mM EDTA, 0.5M NH₂OH.HCl, at pH 7.5 was added with mixing. The reaction was allowed to set for 1.5 hours at ambient temperature. The deprotected antibody was gel filtered through a desalting column into the original buffer to remove unreacted reagents.

Example 12. Improvement of Coating Properties of Thiolated Monoclonal Anti-HIV p24.

Gold sol was prepared as described in Example 1 except that separate aliquots of the sol were adjusted to pH 6, 7, 8 and 9 with K₂CO₃. In parallel, separate aliquots of the underivatized and derivatized monoclonal anti-HIV p24 (see Example 11) were adjusted to 250, 200, 150, 100 and 50 µg/ml. For each of the above, the relative ability of derivatized and underivatized antibody to protect the gold sol from salt-induced agglomeration was made exactly as described in Example 9 (see Figures 7 and 8).

It is apparent from Figures 7 and 8 that the thiolation of this monoclonal antibody resulted in improved coating characteristics as judged by a) the fact that the derivatized antibody did not spontaneously agglomerate the sol, b) the decreased dependence of antibody protection against sol agglomeration on pH, and c) the lower relative amounts of derivatized antibody required to protect from salt-induced sol agglomeration in the presence of salt as compared to the underivatized antibody sol.

Claims

1. Microparticles comprising gold sol coated with at least one compound selected from the group consisting of alkanethiol, alkanethiol derivatives, low molecular weight di-thiol and tri-thiol compounds.
2. Microparticles according to claim 1, wherein said compound is at least one alkanethiol.
3. Microparticles according to claim 1, wherein said compound is a mixture of at least one alkanethiol and at least one alkanethiol derivative.
4. Microparticles according to claim 1, wherein said alkanethiol is a n-alkanethiol of the formula CH₃(CH₂)_nSH, and n equals a whole number from 9 to 23 inclusive.
5. Microparticles according to claim 1, wherein said alkanethiol derivative comprises the formula RCH₂(CH₂)_nSH, where R is selected from the group consisting of OH, COOH, CHO, SH and NH₂, and n is 9 to 23.
6. Microparticles according to claim 1, wherein said compound is a low molecular weight di-thiol compound.
7. Microparticles according to claim 1, wherein said compound is a low molecular weight tri-thiol compound.
8. Microparticles according to claim 1, wherein said gold sol has a diameter of from 10nm to 150nm.
9. Microparticles according to claim 1 which are resistant to salt-induced agglomeration, whereby no more than 10% of the optical density at 540 nm of a suspension of said sol is lost after 10 minutes in the presence of 1.0% NaCl.
10. Microparticles according to claim 1, wherein said microparticles are covalently linked to a binding moiety.
11. Microparticles according to claim 10, wherein the binding moiety is selected from the group consisting of proteins, carbohydrates, antigens, antibodies, ligands, polymers and monomers.
12. Microparticles according to claim 1, additionally comprising a bridging compound which is covalently attached to said compound and to which is covalently linked a binding moiety.
13. Microparticles according to claim 1, wherein additionally a binding moiety is passively adsorbed.

14. A process for coating gold sol microparticles comprising:
- a) mixing gold sol microparticles with an alcohol solution of at least one compound selected from the group consisting of alkanethiol, alkanethiol derivatives, low molecular weight di-thiol and tri-thiol compounds thereby producing coated gold sol microparticles;
 - b) centrifuging said coated sol; and
 - c) resuspending the coated gold sol in buffer.
15. A process for coating gold sol microparticles according to claim 14, wherein said alcohol solution additionally contains surfactant.
16. A process for coating gold sol microparticles according to claim 15, wherein said compound is a mixture of alkanethiol and alkanethiol derivatives.
17. A process for attaching a binding moiety to gold sol microparticles comprising:
- a) coating laid gold sol according to claim 14 to produce a coated gold sol;
 - b) incubating said coated gold sol and binding moiety to attach the binding moiety to said coated sol;
 - c) blocking nonspecific sites on the coated gold sol of step b;
 - d) centrifuging said coated gold sol of step c; and
 - e) resuspending the coated gold sol of step d in buffer.
18. A process for attaching a binding moiety to gold sol microparticles comprising:
- a) coating gold sol according to the process of claim 15;
 - b) incubating a mixture of the gold sol, appropriate chemical crosslinkers and binding moiety to attach the binding moiety to the coated gold sol;
 - c) quenching said mixture;
 - d) blocking nonspecific sites on the coated gold sol of step b;
 - e) centrifuging the coated gold sol of step d; and
 - f) resuspending in buffer.
19. A process for attaching a binding moiety to gold sol microparticles comprising:
- a) coating gold sol microparticles according to the process of claim 16;
 - b) incubating a mixture of said coated gold sol, appropriate chemical crosslinkers and binding moiety to attach the binding moiety to the coated gold sol;
 - c) quenching said mixture;
 - d) blocking nonspecific sites on said gold sol of step b;
 - e) centrifuging said gold sol of step d; and
 - f) resuspending in buffer.
20. A process for attaching a binding moiety to gold sol microparticles comprising:
- a) coating gold sol microparticles according to the process of claim 15;
 - b) incubating said coated gold sol with a binding moiety;
 - c) blocking nonspecific sites on said coated gold sol of step b;
 - d) centrifuging said coated gold sol of step c;
 - e) resuspending said gold sol of step d in buffer.
21. A process for attaching a binding moiety to gold sol microparticles comprising:
- a) modifying said binding moiety by chemical thiolation;
 - b) incubating the thiolated binding moiety of step a with gold sol microparticles;
 - c) blocking nonspecific sites on the gold sol of step b;
 - d) centrifuging the gold sol of step c; and
 - e) resuspending the gold sol of step d in the buffer.

22. A diagnostic kit for use in immunoassays comprising microparticles according to claim 1, and a buffer solution.

Patentansprüche

5

1. Mikropartikel mit Gold-Sol, die mit mindestens einer Verbindung der aus folgenden Stoffen bestehenden Gruppe beschichtet sind
Alkanthiol, Alkanthiolderivate, Dithiol- und Trithiolverbindungen mit geringem Molekulargewicht.

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2. Mikropartikel gemäss Anspruch 1, worin die genannte Verbindung mindestens ein Alkanthiol ist.

3. Mikropartikel gemäss Anspruch 1, worin die genannte Verbindung eine Mischung aus mindestens einem Alkanthiol und mindestens einem Alkanthiolderivat ist.

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4. Mikropartikel gemäss Anspruch 1, worin das genannte Alkanthiol ein n-Alkanthiol der Formel $\text{CH}_3(\text{CH}_2)_n\text{SH}$ ist und n eine Ganzzahl von 9 bis und mit 23 ist.

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5. Mikropartikel gemäss Anspruch 1, worin das genannte Alkanthiolderivat die Formel $\text{RCH}_2(\text{CH}_2)_n\text{SH}$ umfasst, in der R aus der aus OH, COOH, CHO, SH und NH_2 bestehenden Gruppe ausgewählt ist, und n eine Ganzzahl von 9 bis 23 ist.

6. Mikropartikel gemäss Anspruch 1, worin die genannte Verbindung eine Dithiol-Verbindung mit geringem Molekulargewicht ist.

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7. Mikropartikel gemäss Anspruch 1, worin die genannte Verbindung eine Trithiol-Verbindung mit geringem Molekulargewicht ist.

8. Mikropartikel gemäss Anspruch 1, worin das genannte Gold-Sol einen Durchmesser von 10 nm bis 150 nm aufweist.

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9. Mikropartikel gemäss Anspruch 1, die gegen salzinduzierte Agglomeratbildung resistent ist, wobei in Anwesenheit von 1,0 % NaCl nach 10 Minuten nicht mehr als 10 % der optischen Dichte bei 540 nm einer Suspension des genannten Sols verloren geht.

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10. Mikropartikel gemäss Anspruch 1, worin die genannten Mikropartikel kovalent mit einem Verbindungsteil verbunden sind.

11. Mikropartikel gemäss Anspruch 10, worin das Verbindungsteil aus einer aus Proteinen, Kohlenhydraten, Antigenen, Antikörpern, Liganden, Polymeren und Monomeren bestehenden Gruppe ausgewählt wird.

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12. Mikropartikel gemäss Anspruch 1, die zusätzlich eine Brückenverbindung umfassen, die kovalent mit der genannten Verbindung verbunden und an die kovalent ein Verbindungsteil gebunden ist.

13. Mikropartikel gemäss Anspruch 1, worin zusätzlich ein Verbindungsteil passiv adsorbiert wird.

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14. Verfahren zur Beschichtung von Gold-Sol-Mikropartikeln umfassend:

a) Mischen von Gold-Sol-Mikropartikeln mit einer Alkohollösung bestehend aus mindestens einer Verbindung, die aus einer aus Alkanthiol, Alkanthiolderivaten, Dithiol- und Trithiol-Verbindungen mit geringem Molekulargewicht bestehenden Gruppe ausgewählt wird, wodurch beschichtete Gold-Sol-Mikropartikel hergestellt werden;

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b) Zentrifugation des genannten beschichteten Sols; und

c) Resuspendierung des beschichteten Gold-Sols in Pufferlösung.

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15. Verfahren zur Beschichtung von Gold-Sol-Mikropartikeln gemäss Anspruch 14, worin die genannte Alkohollösung zusätzlich einen oberflächenaktiven Stoff enthält.

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16. Verfahren zur Beschichtung von Gold-Sol-Mikropartikeln gemäss Anspruch 15, worin die genannte Verbindung eine Mischung von Alkanthiol und Alkanthiolderivaten ist.

17. Verfahren zur Bindung eines Verbindungsteils an Gold-Sol-Mikropartikel, umfassend:

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- a) Beschichtung des genannten Gold-Sols gemäss Anspruch 14, um beschichtetes Gold-Sol herzustellen;
- b) Inkubieren des genannten Gold-Sols und des Verbindungsteils, um das Verbindungsteil an das genannte beschichtete Sol zu binden;
- 10 c) Blockieren nichtspezifischer Stellen auf dem beschichteten Gold-Sol von Schritt b);
- d) Zentrifugieren des unter Schritt c) genannten beschichteten Gold-Sols;
- 15 e) Resuspendieren in einer Pufferlösung des in Schritt d) genannten beschichteten Gold-Sols.

18. Verfahren zur Bindung eines Verbindungsteils an Gold-Sol-Mikropartikel, umfassend:

20

- a) Beschichten von Gold-Sol gemäss Verfahren unter Anspruch 15;
- b) Inkubieren einer Mischung des Gold-Sols, eines geeigneten chemischen Vernetzungsmittels und des Verbindungsteils, um das Verbindungsteil an das beschichtete Gold-Sol zu binden;
- 25 c) Abschrecken der genannten Mischung;
- d) Blockieren nichtspezifischer Stellen auf dem beschichteten Gold-Sol von Schritt b);
- e) Zentrifugieren des unter Schritt d) genannten beschichteten Gold-Sols; und
- 30 f) Resuspendierung in einer Pufferlösung.

19. Verfahren zur Bindung eines Verbindungsteils an Gold-Sol-Mikropartikel, umfassend:

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- a) Beschichtung von Gold-Sol-Mikropartikeln gemäss Verfahren unter Anspruch 16;
- b) Inkubieren einer Mischung des genannten beschichteten Gold-Sols, eines geeigneten chemischen Vernetzungsmittels und eines Verbindungsteils, um das Verbindungsteil an das beschichtete Gold-Sol zu binden;
- 40 c) Abschrecken der genannten Mischung;
- d) Blockieren nichtspezifischer Stellen auf dem genannten Gold-Sol von Schritt b);
- e) Zentrifugieren des unter Schritt d) genannten Gold-Sols; und
- 45 f) Resuspendierung in einer Pufferlösung.

20. Verfahren zur Bindung eines verbindungsteils an Gold-Sol-Mikropartikel, umfassend:

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- a) Beschichten von Gold-Sol-Mikropartikeln Verfahren unter Anspruch 15;
- b) Inkubieren des genannten beschichteten Gold-Sols mit einem Verbindungsteil;
- c) Blockieren nichtspezifischer Stellen auf dem unter Schritt b) genannten beschichteten Gold-Sol;
- 55 d) Zentrifugieren des unter Schritt c) genannten beschichteten Gold-Sols;
- e) Resuspendierung des unter Schritt d) genannten beschichteten Gold-Sols in einer Pufferlösung.

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21. Verfahren zur Bindung eines Verbindungsteils an Gold-Sol-Mikropartikel, umfassend:

- a) Verändern des genannten Verbindungsteils durch chemische Thiolierung;
- b) Inkubieren des in Schritt a) genannten thiolierten Verbindungsteils mit Gold-Sol-Mikropartikeln;
- c) Blockieren nichtspezifischer Stellen auf dem in Schritt b) genannten Gold-Sol;
- d) Zentrifugieren des unter Schritt c) genannten Gold-Sols; und
- e) Resuspendierung in einer Pufferlösung des unter Schritt d) genannten Gold-Sols.

22. Ein Diagnostik-Set für Immunassays umfassend Mikropartikel gemäss Anspruch 1 sowie eine Pufferlösung.

Revendications

1. Microparticules comprenant un sol d'or revêtu d'au moins un composé choisi dans le groupe consistant en alcanethiol, dérivés d'alcanethiol, dérivés de dithiol et de trithiol de faible poids moléculaire.
2. Microparticules selon la revendication 1, dans lesquelles ledit composé est au moins un alcanethiol.
3. Microparticules selon la revendication 1, dans lesquelles ledit composé est un mélange d'au moins un alcanethiol et d'au moins un dérivé d'alcanethiol.
4. Microparticules selon la revendication 1, dans lesquelles ledit alcanethiol est un n-alcanethiol de formule $\text{CH}_3(\text{CH}_2)_n\text{SH}$, et n est un nombre entier de 9 à 23, bornes comprises.
5. Microparticules selon la revendication 1, dans lesquelles ledit dérivé d'alcanethiol comprend les dérivés de formule $\text{RCH}_2(\text{CH}_2)_n\text{SH}$, où R est choisi dans le groupe consistant en OH, COOH, CHO, SH et NH_2 et n est un nombre de 9 à 23.
6. Microparticules selon la revendication 1, dans lesquelles ledit composé est un dérivé de dithiol de faible poids moléculaire.
7. Microparticules selon la revendication 1, dans lesquelles ledit composé est un dérivé de trithiol de faible poids moléculaire.
8. Microparticules selon la revendication 1, dans lesquelles ledit sol d'or présente un diamètre de 10 nm à 150 nm.
9. Microparticules selon la revendication 1, qui sont résistantes à l'agglomération induite par le sel, dans lesquelles pas plus de 10 % de la densité optique à 540 nm d'une suspension dudit sol est perdue après 10 minutes en présence de NaCl à 1,0 %.
10. Microparticules selon la revendication 1, dans lesquelles lesdites microparticules sont liées par covalence à une espèce liante.
11. Microparticules selon la revendication 10, dans lesquelles la partie liante est choisie dans le groupe consistant en protéines, hydrates de carbone, antigènes, anticorps, ligands, polymères et monomères.
12. Microparticules selon la revendication 1, comprenant en outre un composé de pontage qui est fixé de manière covalente audit composé et auquel est liée par covalence une espèce liante.
13. Microparticules selon la revendication 1, dans lesquelles, en outre, une espèce liante est adsorbée passivement.
14. Un procédé de revêtement de microparticules de sol d'or comprenant:
 - a) le mélange des microparticules de sol d'or avec une solution alcoolique d'au moins un composé choisi dans

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- le groupe consistant en alcanethiol, dérivés d'alcanethiol, dithiols et trithiols de faible poids moléculaire, ce qui permet d'obtenir des microparticules de sol d'or revêtues;
b) centrifugation dudit sol d'or revêtu; et
c) remise en suspension du sol d'or revêtu dans un tampon.
- 5
15. Un procédé de revêtement de microparticules de sol d'or selon la revendication 14, dans lequel ladite solution alcoolique contient en outre un agent tensioactif.
- 10
16. Un procédé de revêtement de microparticules de sol d'or selon la revendication 5, dans lequel ledit composé est un mélange d'alcanethiol et de dérivés d'alcanethiol.
17. Un procédé de fixation d'une espèce de liaison à des microparticules de sol d'or comprenant:
- 15
- a) le revêtement du sol d'or selon la revendication 14 pour obtenir un sol d'or revêtu;
b) l'incubation dudit sol d'or revêtu et de la partie de liaison pour fixer la partie de liaison audit sol d'or revêtu;
c) le blocage des sites non spécifiques sur le sol d'or revêtu de l'étape b);
d) centrifugation dudit sol d'or revêtu de l'étape c); et
e) remise en suspension dudit sol d'or revêtu de l'étape d) dans le tampon.
- 20
18. Un procédé de fixation d'une espèce de liaison à des microparticules de sol d'or comprenant:
- a) le revêtement du sol d'or selon le procédé de la revendication 15;
b) l'incubation d'un mélange de sol d'or, d'un agent de réticulation chimique approprié et d'une partie de liaison pour fixer la partie de liaison sur le sol d'or revêtu;
25 c) trempe dudit mélange;
d) blocage des sites non-spécifiques du sol d'or revêtu de l'étape b);
e) centrifugation du sol d'or revêtu de l'étape d); et
f) remise en suspension dans un tampon.
- 30
19. Un procédé de fixation d'une espèce de liaison sur des microparticules de sol d'or comprenant:
- a) le revêtement des microparticules du sol d'or selon le procédé de la revendication 16;
b) l'incubation d'un mélange dudit sol d'or revêtu, d'agents de réticulation chimiques appropriés et de l'espèce de liaison pour fixer l'espèce de liaison au sol d'or revêtu;
35 c) trempe dudit mélange;
d) le blocage des sites non spécifiques dudit sol d'or de l'étape b);
e) centrifugation dudit sol d'or de l'étape d); et
f) remise en suspension dans un tampon
- 40
20. Un procédé de fixation d'une espèce de liaison à des microparticules de sol d'or comprenant:
- a) le revêtement des microparticules du sol d'or selon le procédé de la revendication 15;
b) l'incubation du sol d'or revêtu avec une partie de liaison;
c) le blocage des sites non spécifiques sur ledit sol d'or revêtu de l'étape b);
45 d) centrifugation dudit sol d'or revêtu de l'étape c); et
e) remise en suspension dudit sol d'or de l'étape d) dans un tampon.
21. Un procédé de fixation d'une espèce de liaison sur des microparticules de sol d'or comprenant:
- 50
- a) la modification de l'espèce de liaison par thiolation chimique;
b) incubation de l'espèce de liaison thiolée de l'étape a) par des microparticules de sol d'or;
c) blocage des sites non spécifiques sur le sol d'or de l'étape b);
d) centrifugation du sol d'or de l'étape c);
e) remise en suspension du sol d'or de l'étape d) dans un tampon.
- 55
22. Un kit de diagnostic pour immunoessai comprenant des microparticules selon la revendication 1 et une solution tampon.

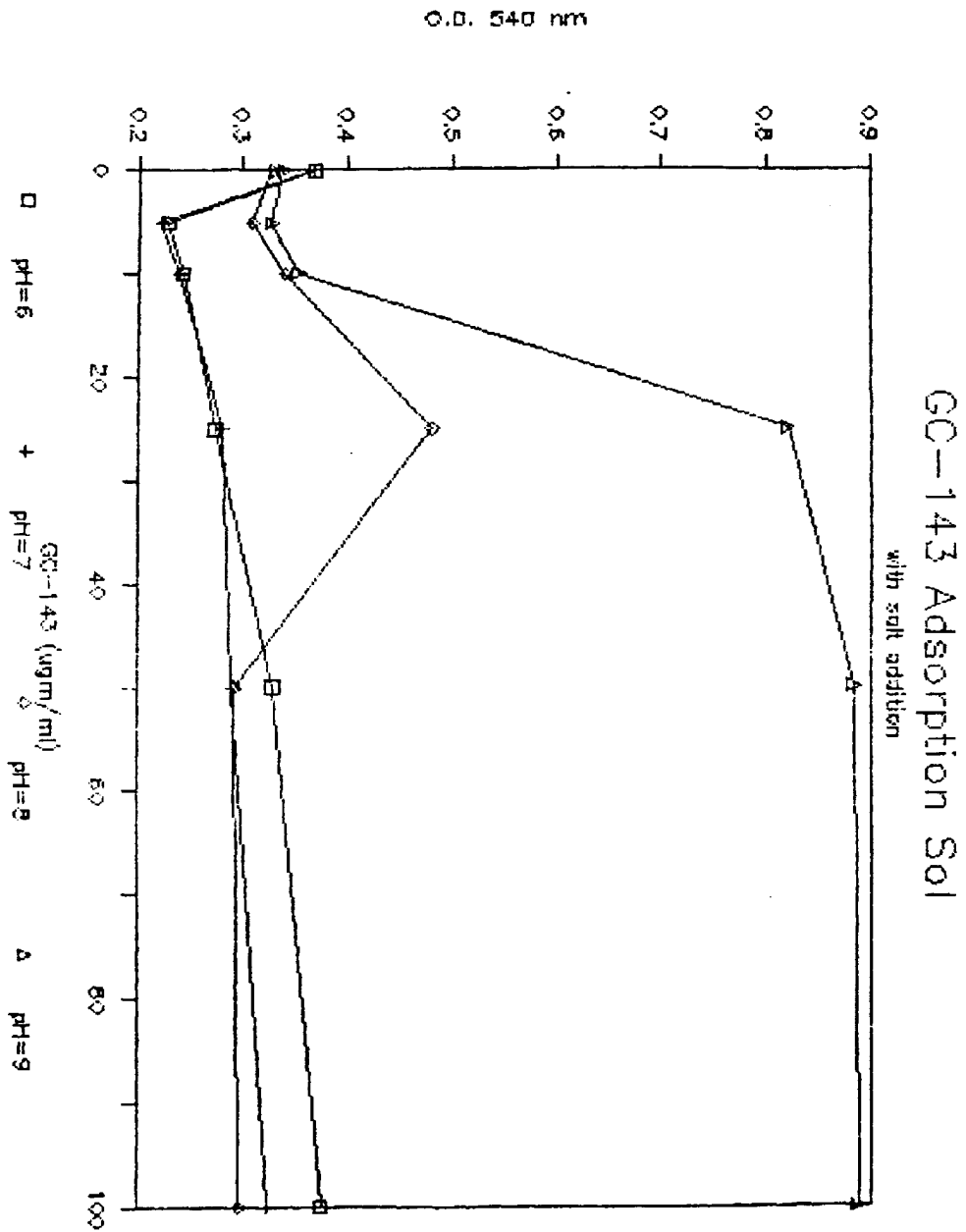


FIGURE 1

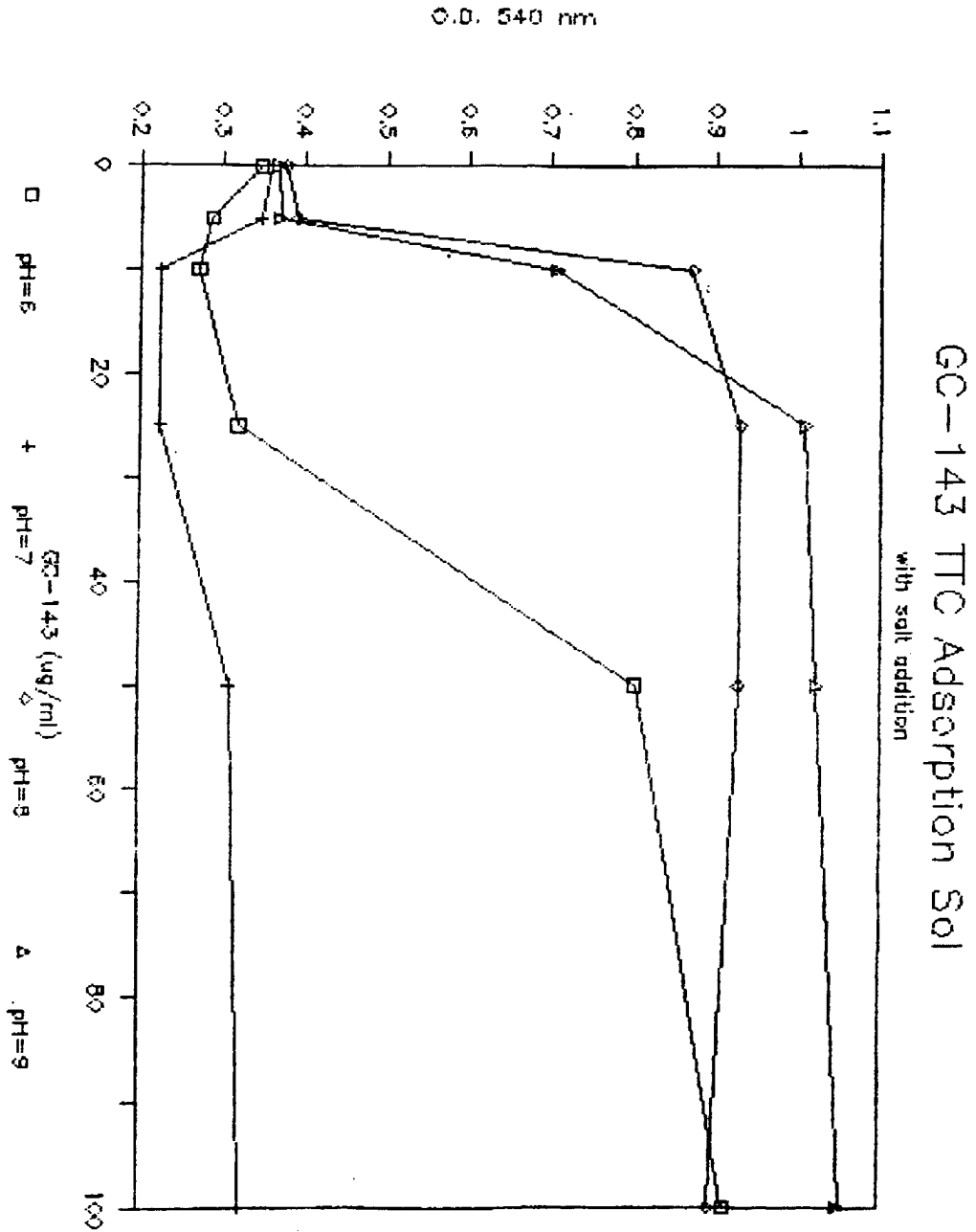


FIGURE 2

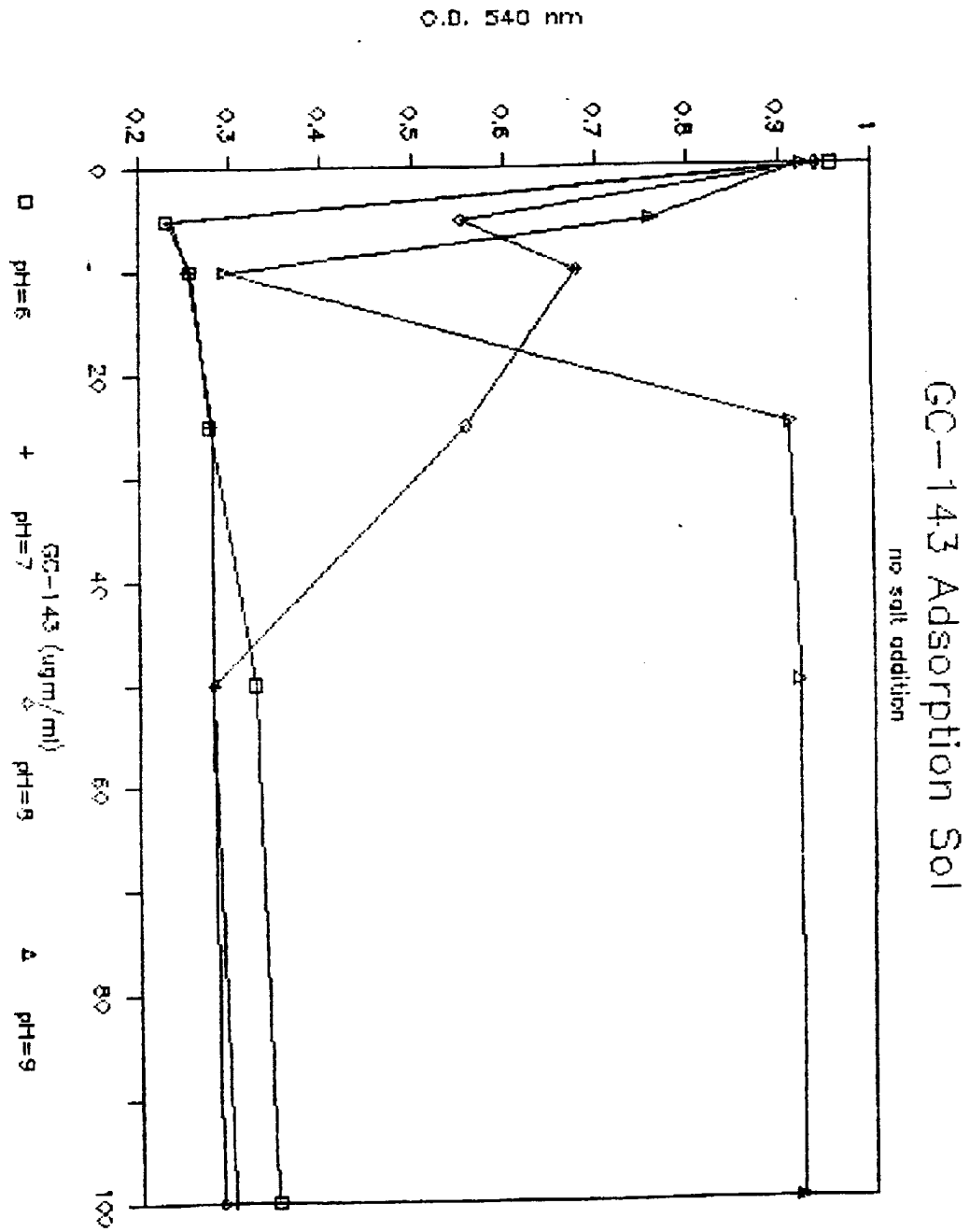


FIGURE 3

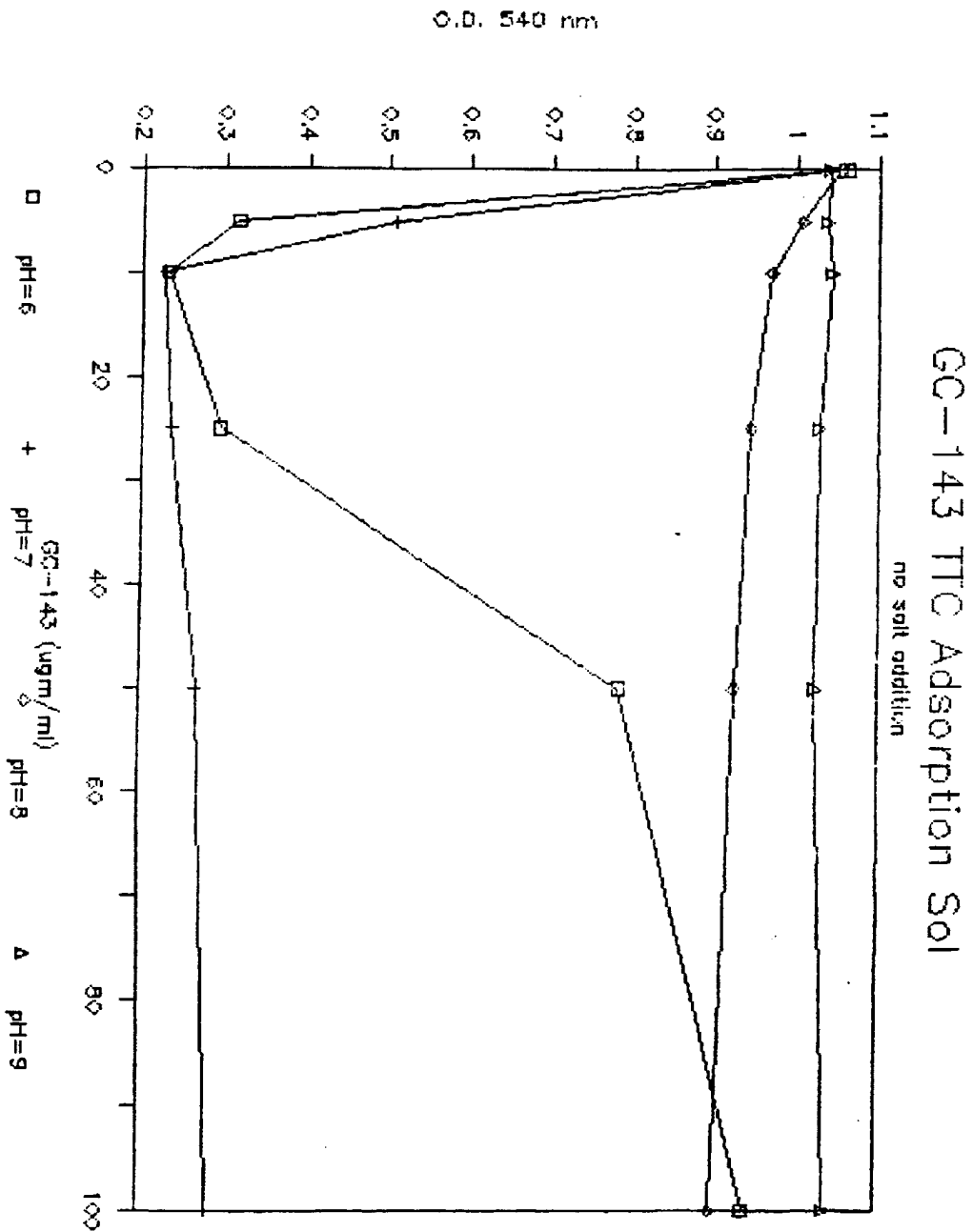


FIGURE 4

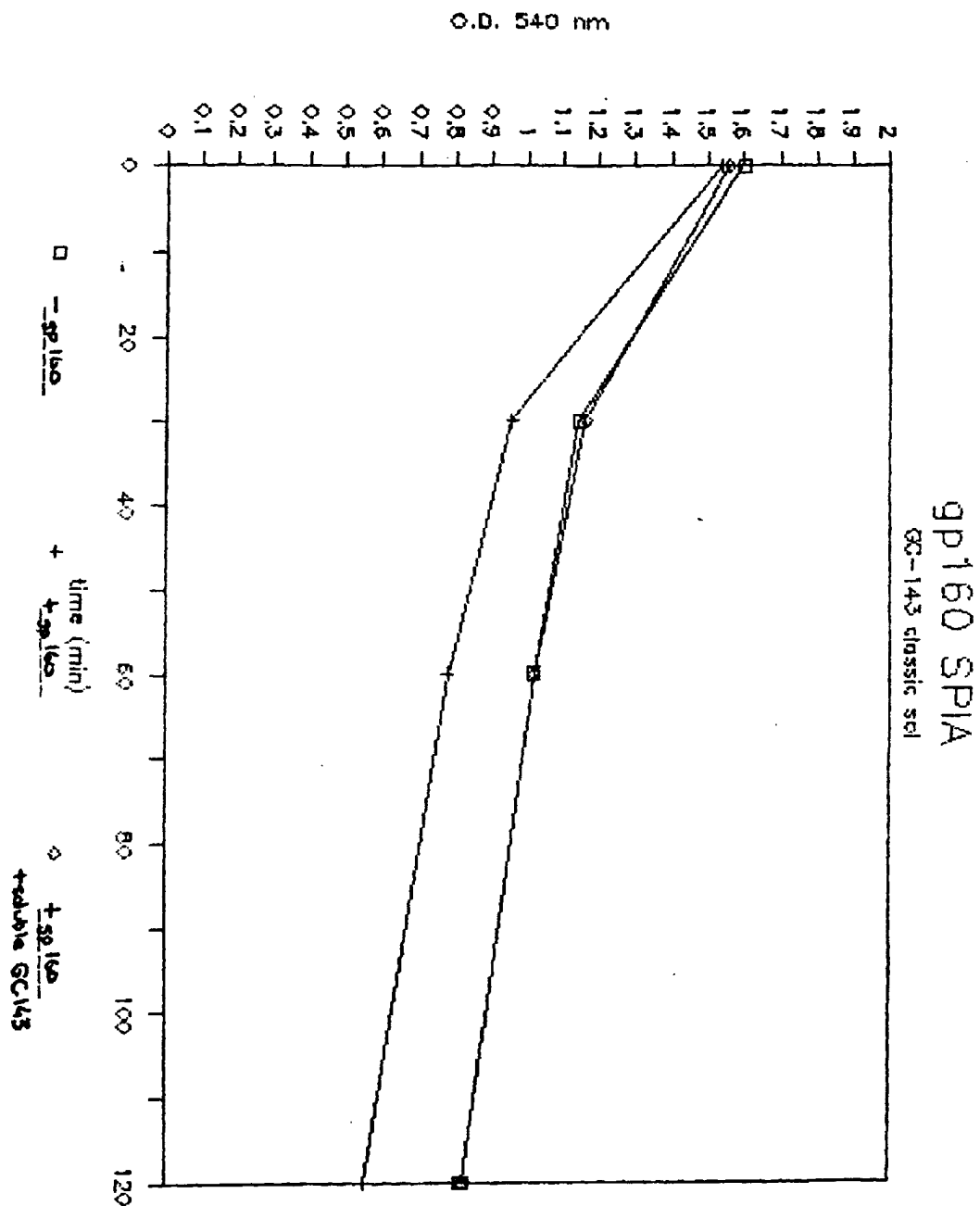


FIGURE 5

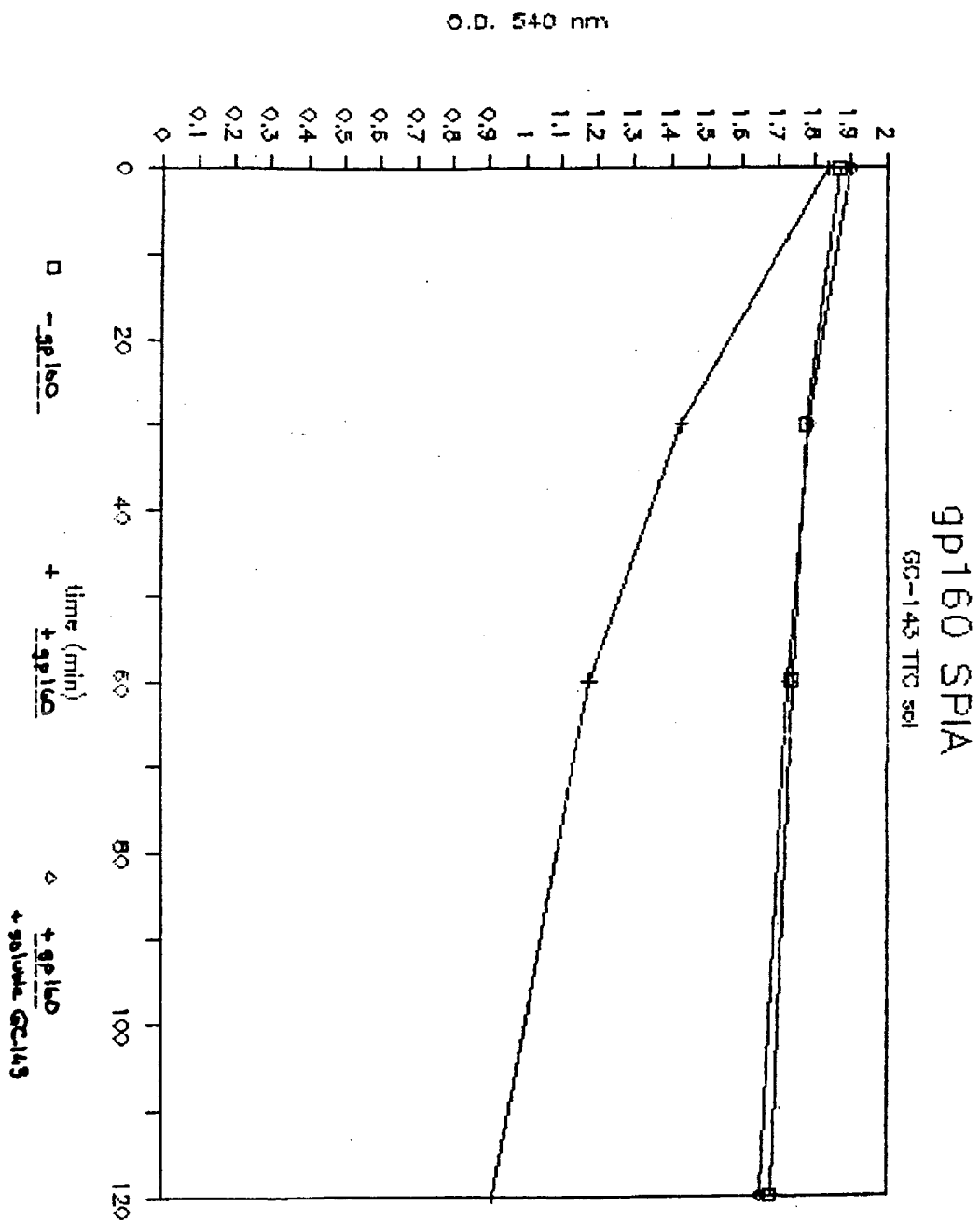


FIGURE 6

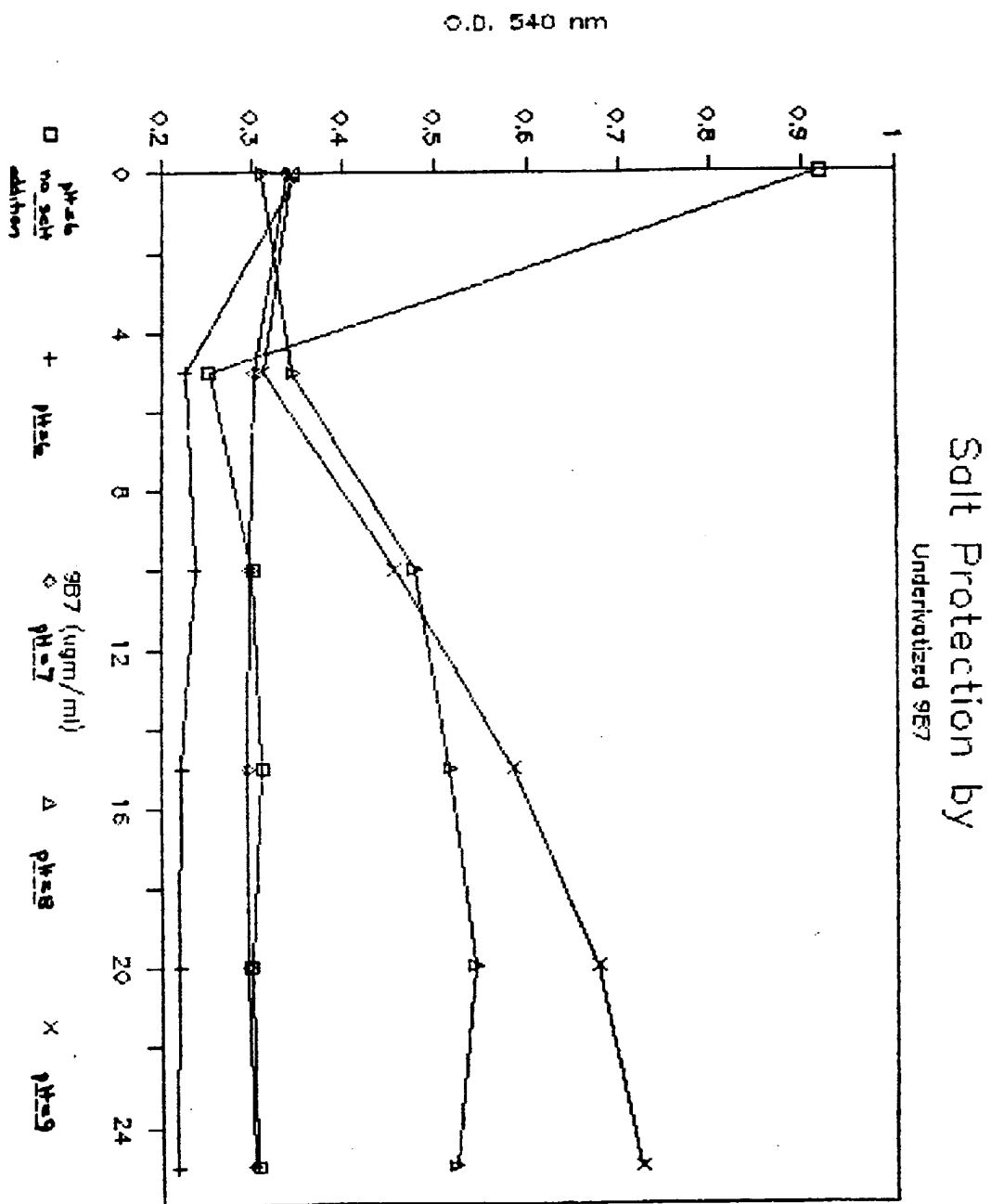


FIGURE 7

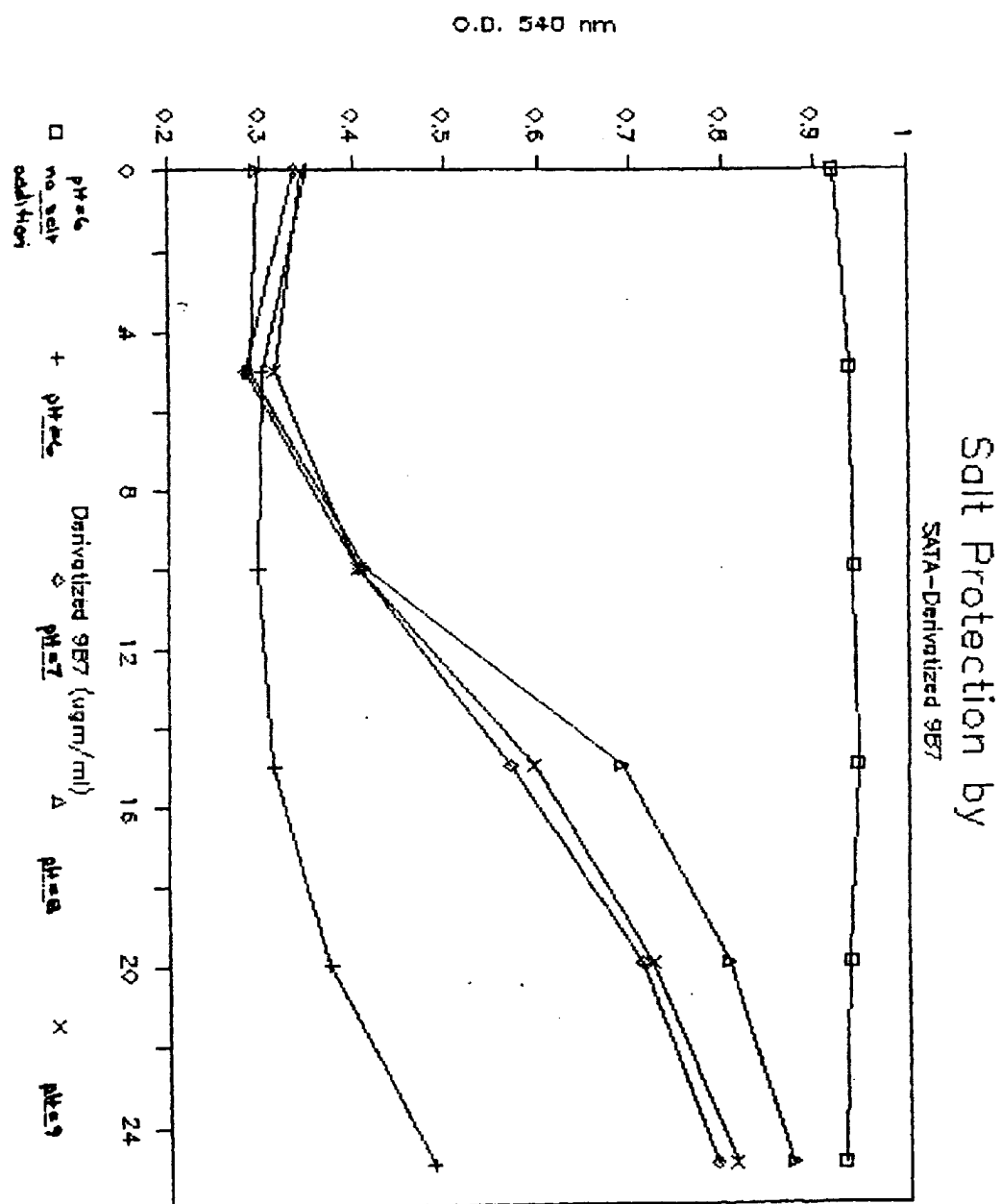


FIGURE 8